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Pulse shape of magnetic fields influences chick embryogenesis*

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INTRODUCTION

Recent studies have demonstrated that biological systems are sensitive to electromagnetic fields (EMF), as shown by changes in growth rate of bacteria (Blackman, Bennane, Weil & Ali, 1975), eucaryote (Adey, 1981; Grundler, Keilmann & Fröhlich, 1977) and tumoural cell cultures (Kim, 1976; Rockwell, 1977), by mutagenic effects (Diebolt, 1978), and by metabolic alterations (Weissbluth, 1979). Most of these studies have been performed using static electromagnetic fields of high intensity or pulsating fields at frequencies above MHz, and results have been contradictory.

Cerebral calcium ion binding (Bawin & Adey, 1976; Bawin, Kaczmarek & Adey, 1975; Bawin, Adey & Sabbot, 1978) and osteogenesis (Bassett, Pawluk & Pilla, 1974*a*, *b*; Bassett, Chiokshi, Hernandez & Pawluk, 1979) are influenced by pulsating fields of very low frequencies, demonstrating 'window' effects at specific frequencies and intensities. Slight modifications of electromagnetic field parameters may have opposite effects; for example, frog erythrocyte dedifferentiation in Ringer solution is inhibited by exposure to 40 and 71 Hz electromagnetic fields, and accelerated with a frequency of 55 Hz (Hinsenkamp, Chiabrera, Pilla & Bassett, 1978).

The waveform appears to be an important factor determining biological responses. When exposed to sinusoidal electrical fields, chick cerebral tissues released a varied amount of preincorporated $^{45}Ca^{2+}$ according to the frequency and intensity of the applied fields. A sinusoidally modulated amplitude caused progressive increase in $^{45}Ca^{2+}$ efflux at modulation frequencies from 6 to 16 Hz, whereas 0·5 and 3·0 Hz did not change this efflux (Bawin *et al.* 1975).

There are few studies on the effects of electromagnetic fields on embryonic development. Levengood (1967) reported that an electromagnetic field of 150 Gauss induced retardation of development in *Drosophila*, and Mulay & Mulay (1964) found that exposure to 100, 600, and 1500 Oe had no effect, although exposure to 3000–4000 Oe for more than one generation resulted in increased abnormalities. This report, however, was not confirmed by Beischer (1964) or by Steen & Ofted (1967).

Studying the effects of electromagnetic fields on amphibian development, Iwasaki, Ohara, Matsumoto & Matsudaira, (1978) reported that the exposure of fertilized or early cleavage eggs of *Xenopus laevis* to a uniform electromagnetic field of 5000 Gauss for up to 72 hours did not affect development or hatchability, while higher intensities of 10000 or more than 17000 Gauss induce pathological disturbances in frog and salamander embryos (Levengood, 1969; Neurath, 1968).

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Veneziano (1965) demonstrated the sensitivity of chick embryos to uniform magnetostatic fields ranging from 1·1 to 31 Gauss. The exposed embryos present a mean blastoderm diameter smaller than in controls, as well as developmental anomalies. Exposure to the strongest electromagnetic field induces central nervous system defects in 9 % of the embryos, including hyperplasia and general growth retardation. Histological examination reveals cellular disorganization and extra-neural tube formation.

Significant effects on chick development have also been described by Joshi, Khan & Damle (1978) in 25 embryos which were exposed, *in vitro* and at room temperature, to a vertical homogeneous magnetic field of 5000 Oe for 1 hour and then returned to the incubator for 24 hours. All embryos show abnormalities in the nervous system, heart, and somites, and generally retarded development, as well as an increased number of dividing cells in the neuroepithelium.

In a previous paper (Delgado, Leal, Monteagudo & Garcia Gracia, 1982), we described the effects on chick embryos of low frequency electromagnetic fields of 10, 100 and 1000 Hz with intensities of 0·12, 1·2 and 12 microTeslas, (μT) showing the different sensitivity of developing organs to specific frequencies and intensities.

In the present study these investigations have been continued, paying special attention to the differential embryological effects of pulse shape.

MATERIALS AND METHODS

A total of 659 freshly fertilized White Leghorn hen eggs was used (295 exposed to electromagnetic fields and 364 unexposed controls). Pairs of eggs were placed inside cylindrical coils 170 mm in length and 68·6 mm in diameter, each made of 1000 turns of 0·33 mm enamelled copper wire. Eggs and coils were kept at 38 °C for 48 hours inside Memmert incubators. Additional experiments were performed placing groups of 12 eggs between two square Helmholtz coils 30 cm long, 20 cm apart, and made of 30 turns of 1·5 mm enamelled copper wire. These eggs and coils were also placed in Memmert incubators.

Electrical current to produce electromagnetic fields in the coils was provided by an SD-9 or S-44 Grass stimulator set to deliver biphasic stimulation with zero net value. Uniformity and strength of the electromagnetic field were tested with a Gaussmeter model 750 AR with accessory 1D75 (RFL Industries, Boonton, N.Y). The electromagnetic field did not vary more than 2 % in different coils in series, and when calculated for 1 μT, the energy changes due to eddy currents were less than $3{\cdot}3 \times 10^{-15}$ watts. Since the eggs were stationary, they did not cause spurious electrical fields.

Electrical current parameters used to activate the coils were monitored by a Tektronic 5113 oscilloscope. Repetition rate was always 100 Hz and pulse duration was 500 μsec. As explained later, electromagnetic field intensity varied between 0·4 and 104 μT. The following types of pulse shape were tested:

Pulse A

Rise and fall times were 100 μsec with a declining plateau, with some ripples in the curve, and a long duration post-pulse negative amplitude, as shown in Figure 1 A. The pulse was generated by a Grass SD-9 stimulator feeding five cylindrical coils connected in series, permitting simultaneous experiments with five pairs of eggs.

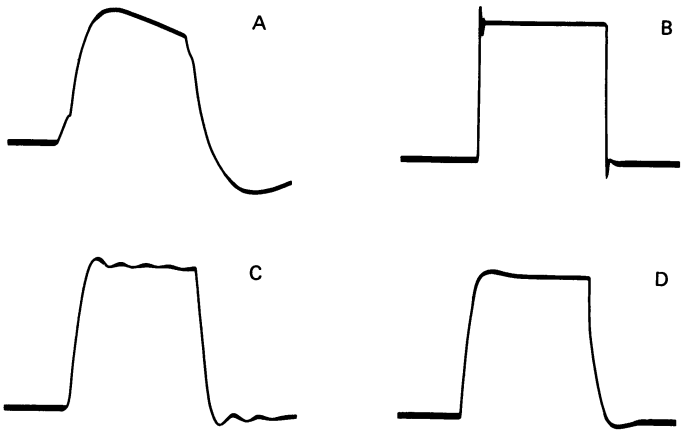


Fig. 1. Pulse signals: In all experiments, pulse duration (500 μsec) and repetition rate (100 Hz) were constant. Four different pulse shapes were used: (A) rise time 100 μsec; (B) rise time 2 μsec; (C) rise time 42 μsec; (D) rise time 42 μsec.

Pulse B

Rise and fall times were 2 μsec, as shown in Figure 1B. The pulse was generated by a Grass SD-9 stimulator feeding two square Helmholtz coils in parallel. The low resistance permitted rapid transit of current with great uniformity of pulses.

Pulse C

Rise and fall times were 42 μsec with several ripples in the curve (Fig. 1C). The pulse was generated by a Grass S-44 stimulator feeding five cylindrical coils as in Pulse A.

Pulse D

This pulse was similar to pulse C but without ripples. It was generated by a Grass S-44 stimulator feeding only two cylindrical coils (Fig. 1D).

Study of embryos

After 48 hours of incubation the eggs were opened and embryos were immersed in Tyrode solution and then fixed in Carnoy's solution (60% absolute ethanol, 30% chloroform, 10% glacial acetic acid). Gross examination of embryos was performed at × 30 magnification with a Nikon binocular stereomicroscope, and pictures were taken with a photographic attachment. The developmental stage of each embryo was determined according to the Hamburger & Hamilton (1951) scale. The cephalic nervous system, truncal nervous system, heart, extra-embryonic vascularisation, and somites were studied in detail for possible abnormalities.

Histological examination of the embryos was performed by dehydrating samples through an alcohol series and embedding them in paraffin. Transverse sections 7 μm thick were cut serially using a rotary Leitz microtome. Sections were mounted, stained with periodic acid–Schiff (Luna, 1968) and alcian blue at pH 2·5 (Gabe, 1968), and counterstained with haematoxylin. Preparations were studied with a Nikon Apophot microscope. Photographs were taken with a 35 mm Nikon camera.

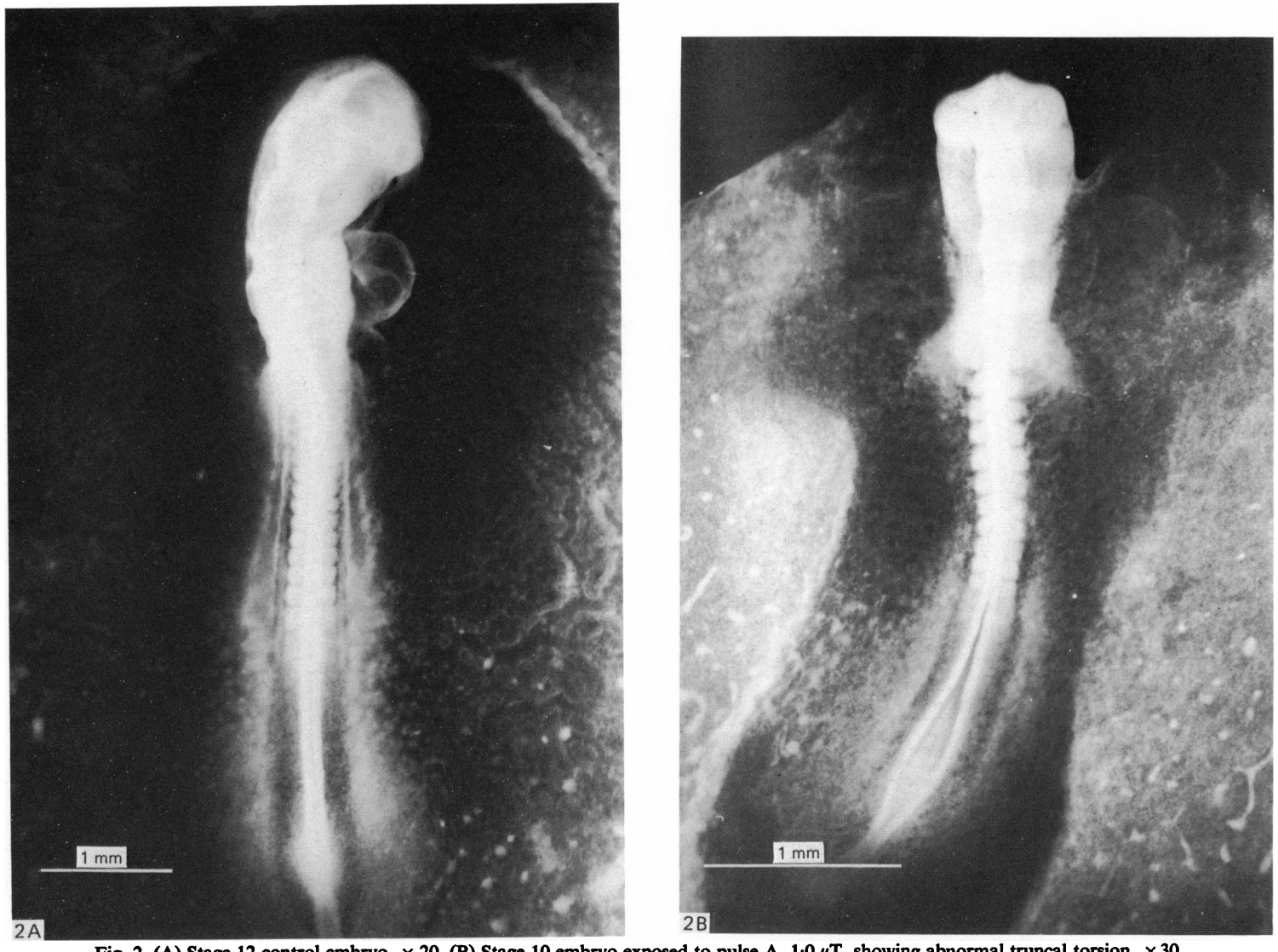


Fig. 2. (A) Stage 12 control embryo. × 20. (B) Stage 10 embryo exposed to pulse A, 1·0 μT, showing abnormal truncal torsion. × 30.

RESULTS

Observation of each embryo with the stereomicroscope permitted classification of its stage of development according to the Hamburger–Hamilton scale. Stage 11 was characterised as follows: cephalic torsion to the right was clearly visible and the cephalic nervous system showed five primitive regions. The division of the prosencephalon into telencephalon and diencephalon had begun, and the rhombencephalon was segmented into 6–7 monomeric regions, the first to become the metencephalon and the others to become the myelencephalon. At this stage, the anterior neuropore was closed and the optic vesicles were constricted at the base. The auditory pits were wide open. The heart was located at the right of the embryo and the extra-embryonic vessels were well developed. There were 13–15 somite pairs.

At the end of stage 12 (Fig. 2A), cephalic flexion was almost complete and torsion was detectable as far as the fifth or sixth somite pair. The five cerebral vesicles were clearly differentiated and there were 19 somite pairs. The heart, in 'S' shape, was beating and primitive blood was circulating between the embryo and the extra-embryonic zone. Extra-embryonic vascularisation was well developed; the two anterior vitelline veins were visible as well as the sinus vein at the base of the heart, formed by fusion of the two omphalomesenteric veins.

In our study, histological examination of the embryos was concentrated on the five systems (cephalic and truncal nervous systems, heart, extra-embryonic vascularisation and somites).

Classification of embryos

According to the Hamburger–Hamilton scale, normal embryos should reach stage 12 after 48 hours of incubation. We therefore placed the lower limit of normality at stage 9 which corresponds to about threequarters of the total development time. Embryos which had reached at least stage 9 and exhibited normal morphological and histological characteristics in the five systems were classified as *normal.* Embryos which had not reached stage 9, representing a delay of at least 11 hours in their development, or those which presented morphological and/or histological alterations in any of the five systems, were classified as *abnormal.* In this group, sub-embryos which had not reached stage 4 (the definitive primitive streak stage) were classified as 'non-developed'.

Spontaneous abnormalities varied among the lots of eggs, as demonstrated by comparing the controls in different experiments. Therefore, the significance of experimental results was established by evaluating the simultaneously incubated exposed and control embryos of the same lot. As the control samples had a high frequency of spontaneous abnormalities, they provided a basis for evaluation of detrimental, neutral, or beneficial effects of applied electromagnetic fields.

Null effects of presence of coils on embryonic development

Exposure to electromagnetic fields required the placement of eggs within the coils. Even in the absence of an electromagnetic field, this procedure might modify the uniform temperature in the incubator, constituted a shield for the egg, or introduced unknown elements disturbing embryonic development.

To evaluate these possibilities, 39 eggs were incubated inside cylindrical coils with no electromagnetic field and 43 eggs were simultaneously incubated outside the coils.

Table 1. *Development of embryos lying inside and outside coils in the absence of electromagnetic fields*

Embryos	Inside coils ($N = 39$)	Outside coils ($N = 43$)
Normals		
No.	30	34
%	76·9	79·0
Mean stage	11·8	11·4
Abnormals		
No.	9	9
% (1)	23·0	20·9
Mean stage	7·7	9·1
Non-developed		
No.	2	3
%	5·1	6·9
Abnormality ratio (1) (% inside/% outside)	1·10 ($P \simeq 0{\cdot}75$)	

N, total number of embryos.

Table 2. *Abnormalities of systems of embryos in Table* 1

Systems	Inside coils ($N = 39$)	Outside coils ($N = 43$)
CNS		
Abnormals	8	8
%	20·5	18·6
Ratio	1·10 ($P \simeq 0{\cdot}75$)	
TNS		
Abnormals	7	6
%	17·9	13·9
Ratio	1·28 ($0{\cdot}75 > P > 0{\cdot}50$)	
Heart		
Abnormals	6	6
%	15·3	13·9
Ratio	1·10 ($P \simeq 0{\cdot}75$)	
Vessels		
Abnormals	6	7
%	15·3	16·2
Ratio	0·94 ($P \simeq 0{\cdot}90$)	
Somites		
Abnormals	6	7
%	15·3	16·2
Ratio	0·94 ($P \simeq 0{\cdot}90$)	

CNS, cephalic nervous system; TNS, truncal nervous system; N, total number of embryos.

Table 3. *Normal and abnormal embryos in controls and after exposure to electromagnetic fields, pulse A, at indicated intensities*

	0·4 μT		1·0 μT		10·4 μT		13·9 μT		104 μT	
	Exposed	Controls	Exposed	Controls	Exposed	Controls	Exposed	Controls	Exposed	Controls
Total no. of embryos (*N*)	30	37	55	55	41	41	32	32	59	53
Normal										
No.	21	25	32	42	27	29	15	21	42	40
%	70·0	67·5	58·1	76·3	65·8	70·7	46·8	65·6	71·1	75·4
Mean stage	12·3	12·4	13·0	12·9	11·3	11·3	10·5	11·5	11·6	11·6
Abnormals										
No.	9	12	23	13	14	12	17	11	17	13
% (1)	30·0	32·4	41·8	23·6	34·1	29·2	53·1	34·3	28·8	24·5
Mean stage	10·8	10·5	12·0	10·5	9·4	9·7	8·4	8·1	8·2	8·8
Non-developed										
No.	1	4	2	2	1	1	5	1	3	4
%	3·3	10·8	3·6	3·6	2·4	2·4	15·6	3·1	5·0	7·5
Abnormality ratio (1) % Exposed/% controls	0·92 ($P \simeq 1·0$)		1·77 ($0·10 > P > 0·05$)		1·16 ($0·90 > P > 0·75$)		1·54 ($0·25 > P > 0·10$)		1·17 ($P \simeq 1·0$)	

N, total number of embryos.

Table 4. *Abnormalities in systems of embryos in Table* 3

Systems	0·4 μT		1·0 μT		10·4 μT		13·9 μT		104 μT	
	Exposed ($N = 30$)	Controls ($N = 37$)	Exposed ($N = 55$)	Controls ($N = 55$)	Exposed ($N = 41$)	Controls ($N = 41$)	Exposed ($N = 32$)	Controls ($N = 32$)	Exposed ($N = 59$)	Controls ($N = 53$)
CNS										
Abnormals	7	9	16	12	14	11	16	11	17	13
%	23·3	24·3	29·0	21·8	34·1	26·8	50·0	34·3	28·8	24·5
Ratio	0·95		1·33		1·27		1·45		1·17	
	(0·90 > P > 0·75)		(0·90 > P > 0·75)		(0·75 > P > 0·50)		(0·50 > P > 0·25)		(0·90 > P > 0·75)	
TNS										
Abnormals	7	12	14	8	11	9	15	11	16	11
%	23·3	32·4	25·4	14·5	26·8	21·9	46·8	34·3	27·1	20·7
Ratio	0·71		1·75		1·22		1·36		1·30	
	(0·75 > P > 0·50)		(0·10 > P > 0·05)		($P \simeq$ 0·75)		(0·50 > P > 0·25)		(0·75 > P > 0·50)	
Heart										
Abnormals	4	8	9	9	11	10	17	9	14	13
%	13·3	21·6	16·3	16·3	26·8	24·3	53·1	28·1	23·7	24·5
Ratio	0·61		1·00		1·10		1·88		0·96	
	($P \simeq$ 0·50)		(P = 1)		($P \simeq$ 1)		(0·10 > P > 0·05)		(P = 0·90)	
Vessels										
Abnormals	3	7	14	9	11	9	17	9	14	10
%	10·0	18·9	25·4	16·3	26·8	21·9	53·1	28·1	23·7	18·8
Ratio	0·52		1·55		1·22		1·88		1·26	
	(0·50 > P > 0·25)		(0·50 > P > 0·25)		($P \simeq$ 0·75)		(0·10 > P > 0·05)		($P \simeq$ 0·90)	
Somites										
Abnormals	4	9	9	10	10	10	16	11	14	11
%	13·3	24·3	16 3	18·1	24·3	24·3	50·0	34·3	23·7	20·7
Ratio	0·54		0·90		1·00		1·45		1·14	
	(0·50 > P > 0·25)		($P \simeq$ 1)		(P = 1)		(0·50 > P > 0·25)		(0·90 > P > 0·75)	

CNS, cephalic nervous system; TNS, truncal nervous system; N, number of embryos.

Table 5. *Embryos exposed to pulse A, 1·0 μT: detail of six different experiments*

Experiment	1		2		3		4		5		6		Total	
	Exp	C	Exp	C	Exp	C	Exp	C	Exp	C	Exp	C	Exp	C
No. of embryos	10	9	9	10	10	8	9	9	8	10	9	9	55	55
Normals														
No.	2	4	7	9	7	8	6	7	3	6	7	8	32	42
%	20·0	44·4	77·7	90·0	70·0	100	66·6	77·7	37·5	60·0	77·7	88·8	58·1	76·3
Abnormals														
No.	8	5	2	1	3	0	3	2	5	4	2	1	23	13
% (1)	80·0	55·5	22·2	10·0	30·0	—	33·3	22·2	62·5	40·0	22·2	11·1	41·8	23·6
Abnormality ratio (1) (% Exposed/ % controls)	*1·44*		*2·22*		—		*1·50*		*1·56*		*2·00*		*1·77* (0·10 > *P* > 0·05)	

Exp, exposed embryos; C, controls.

Table 6. *Effect of pulse shape at the indicated intensities (μT)*

Pulse shape	B		C		D	
Intensity	0.4 μT		1·0 μT		1·0 μT	
	Exposed	Controls	Exposed	Controls	Exposed	Controls
Total no. of embryos	24	23	30	29	24	12
Normals						
No.	4	15	19	22	7	10
%	16·6	62·2	63·3	75·8	29·1	83·3
Mean stage	11·7	10·4	12·3	12·5	10·7	11·9
Abnormals						
No.	20	8	11	7	17	2
% (1)	83·3	34·7	36·6	24·1	70·8	16·6
Mean stage	8·8	7·9	11·7	11·7	8·7	Non developed
Non-developed						
No.	4	2	2	0	5	2
%	16·6	8·6	6·6	—	20·8	16·6
Abnormality ratio (1) (% exposed/% controls)	2·40 ($0{\cdot}005 > P$)		1·51 ($0{\cdot}50 > P > 0{\cdot}25$)		4·26 ($0{\cdot}01 > P > 0{\cdot}005$)	

As shown in Table 1, frequencies of abnormal (23 and 20·9 % inside and outside the coils, respectively) and non-developed (5·1 and 6·9 %) embryos were similar in eggs placed inside and outside the coils. The abnormality ratio (the percentage of faults inside expressed in relation to the percentage outside the coils) was 1·10, which indicated that incubation of the eggs inside the coils produced no significant differences. The mean developmental stage reached by normal embryos was also similar when eggs were placed inside and outside the coils (stages 11.8 and 11.4 respectively). The only difference was in the mean developmental stage of abnormal embryos which was slightly earlier in eggs placed inside the coils (stage 7.7) as compared with eggs placed outside the coils (stage 9.1). This difference corresponded to approximately 3 hours of development and was not statistically significant because of the spontaneous variability of abnormal embryos in controls (Tables 3–6).

Analysis of abnormalities present in the five systems (Table 2) revealed no differences between embryos developed inside or outside the inactivated coils. It was therefore concluded that in the absence of an electromagnetic field, placement of eggs inside the coils caused no significant disturbance of early development.

Embryological effects of electromagnetic fields with pulse shape A

With pulse A (Fig. 1) and constant parameters of 100 Hz, 500 μsec pulse duration experiments were performed on 435 eggs (217 exposed and 218 controls) to test the effects of electromagnetic fields with intensities of 0·4, 1·0, 10·4, 13·9, and 104 μT. Data on the general development of embryos are shown in Table 3. Analysis of disturbances in each system is presented in Table 4 which also indicates for each system the ratio of alterations in exposed compared with control embryos. For example, with 0·4 μT intensity, the total number of exposed embryos was 30, and seven (23·3 %) had cephalic nervous system abnormalities. The total number of control embryos in this group was 37, and nine (24·3 %) had cephalic nervous system abnormalities. Therefore, the ratio of these abnormalities at 0·4 μT was 0·95 (23·3 % in exposed: 24·3 % in controls) which was not significant. Calculations were made in the same way for

abnormalities in each system. A ratio above 1·0 suggested that electromagnetic field effects were teratogenic, while a ratio below 1·0 suggested 'beneficial' effects.

Exposure to 0·4 μT

A total of 30 eggs was exposed to an electromagnetic field of 0·4 μT intensity and compared with 37 controls. In both groups, about 70 % of the embryos were normal, developing just beyond stage 12 (Table 3). Abnormal embryos in exposed and control groups (30 and 32·4 % respectively) had a ratio (0·92) which was not significant, and both groups developed to between stages 10 and 11.

Although the analysis of abnormalities in the five systems showed no significant differences (Table 4), there was a beneficial 'trend' in exposed embryos, which had ratios below 1·0 for all developing systems. Possible beneficial effects of the 0·4 μT electromagnetic field were also suggested by the respective frequencies of non-developed embryos in the exposed (3·3 %) and control (10·8 %) groups (Table 3).

Exposure to 1·0 μT

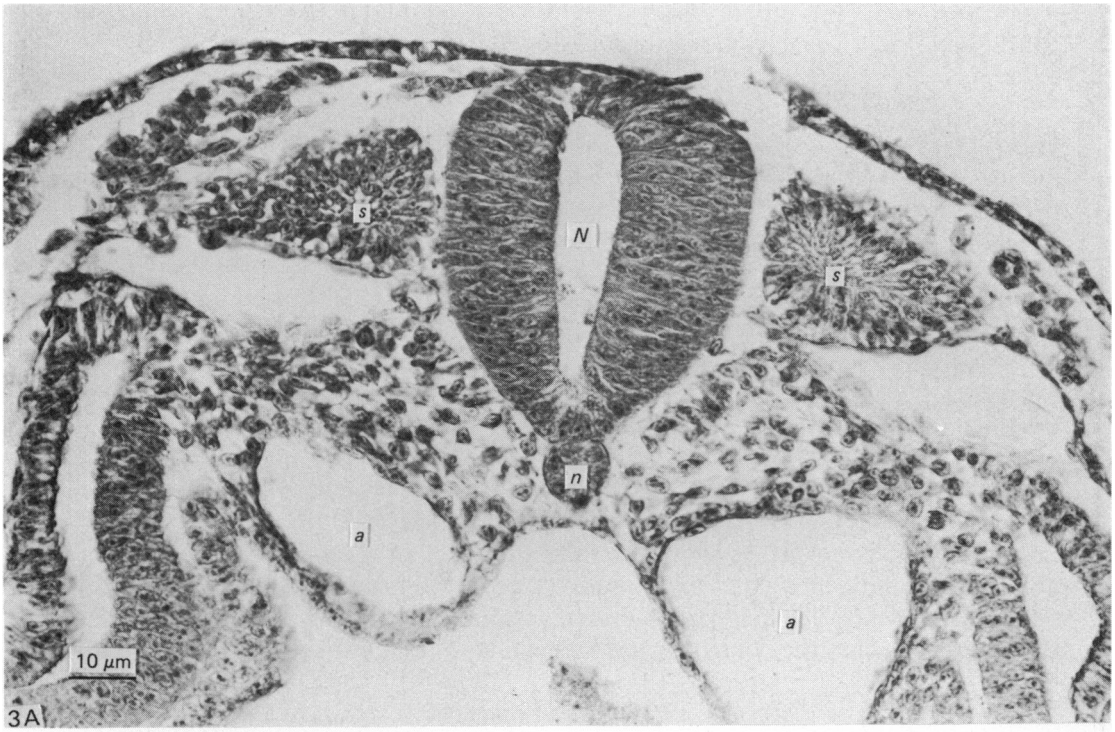
A total of 55 eggs was exposed to an electromagnetic field of 1·0 μT in six experiments, with 55 controls. As shown in Table 3, 41·8 % of the exposed embryos were abnormal, compared with 23·6 % of the controls, the ratio being 1·77 ($0{\cdot}1 > P > 0{\cdot}05$). As shown in Table 5, the number of abnormal embryos was always greater in the exposed than in the control group, indicating a slight but significant teratogenic effect.

Normal embryos reached a similar mean stage of development in exposed eggs (stage 13) and in controls (stage 12.9), while abnormal exposed embryos reached a more advanced stage of development (stage 12.0) than did controls (stage 10.5), this advance corresponding to approximately 12 hours of development (Table 3). A possible explanation might be that spontaneous abnormalities affected general development, while induced abnormalities appeared to be more system-specific, indicating the greater sensitivity of some systems to electromagnetic fields. Abnormalities in the truncal nervous system were more frequent in exposed than in control embryos, whereas cephalic nervous system, heart, vessel, and somite abnormalities were not significantly different (Table 4). Gross examination of the embryos showed that malformations of the neural tube in exposed embryos presented two types of disturbances not found in controls: abnormal torsion of the axis (six cases; Fig. 2B), and abnormally dense folds (five cases). In addition, short neural tubes were less common in abnormal exposed embryos (three out of 14 cases) than in abnormal controls (seven out of eight). An open neural tube was found as often in exposed as in control embryos.

Histological analysis of the abnormal exposed embryos showed a general dorso-ventral flattening of the organs. Compared with controls of the same stage (Fig. 3A), truncal neural tubes were thick and poorly organised, somites were slightly retarded, and alcian blue-stained components were less abundant in the basal membranes of the neuroepithelium, ectoderm, notochordal sheath, and around the somites (Fig. 3B).

Exposure to 10·4 μT

A total of 41 embryos was exposed to an electromagnetic field of 10·4 μT, and development was compared to the same number of controls. The frequency of abnormal embryos was similar (close to 30 %) in both groups with no differences in the developmental stage (Table 3). Ratios of abnormalities in the different systems



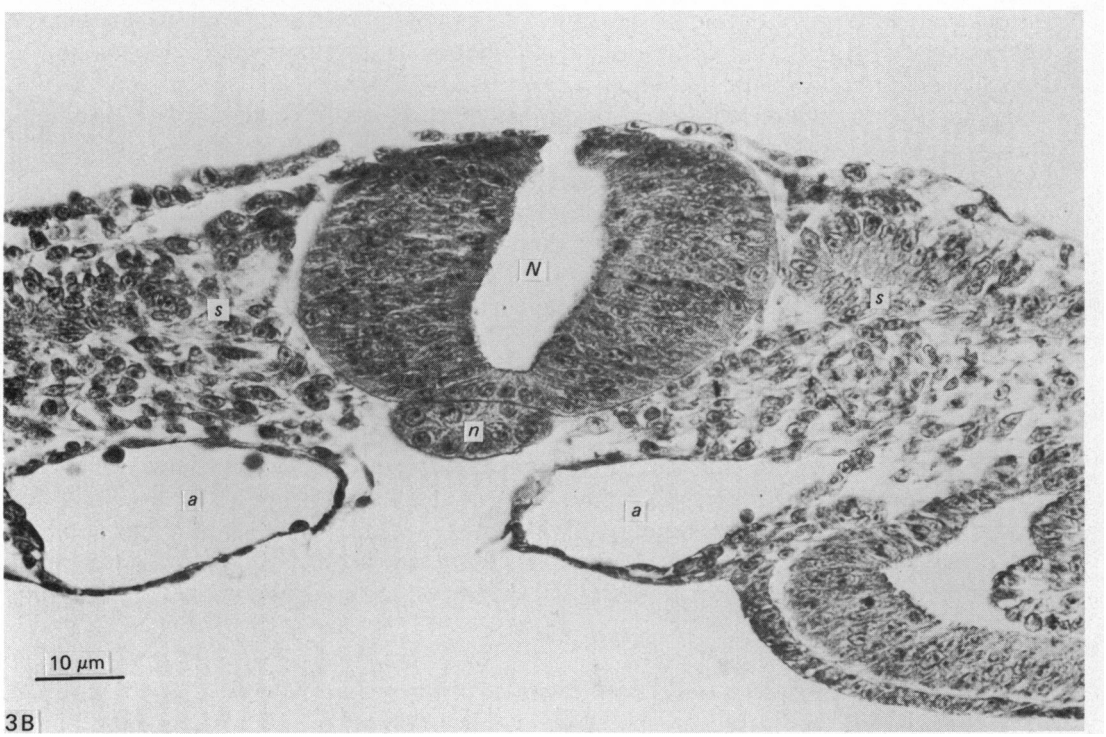
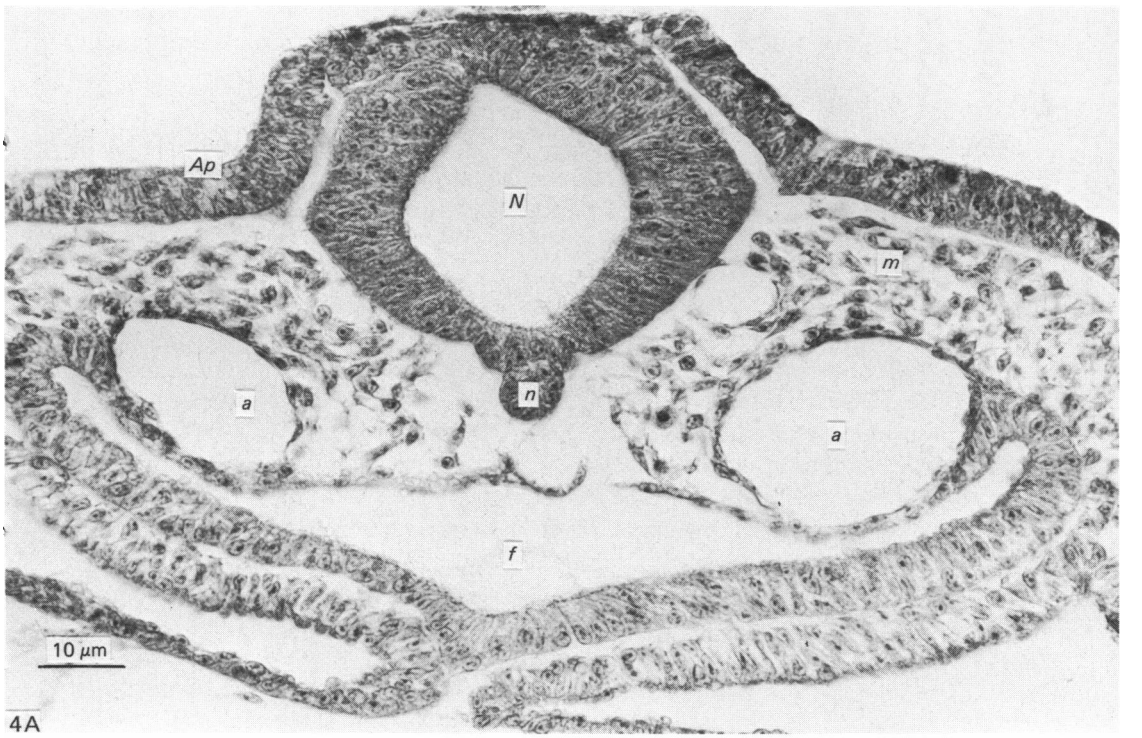


Fig. 3. Transverse sections through the third somite pair. (A) Stage 12 control embryo. (B) Embryo exposed to pulse A, 1·0 μT. There is dorsoventral flattening of all organs and a thick, poorly organised truncal nervous system. *N*, neural tube; *a*, aorta; *s*, somite; *n*, notochord. ×800.



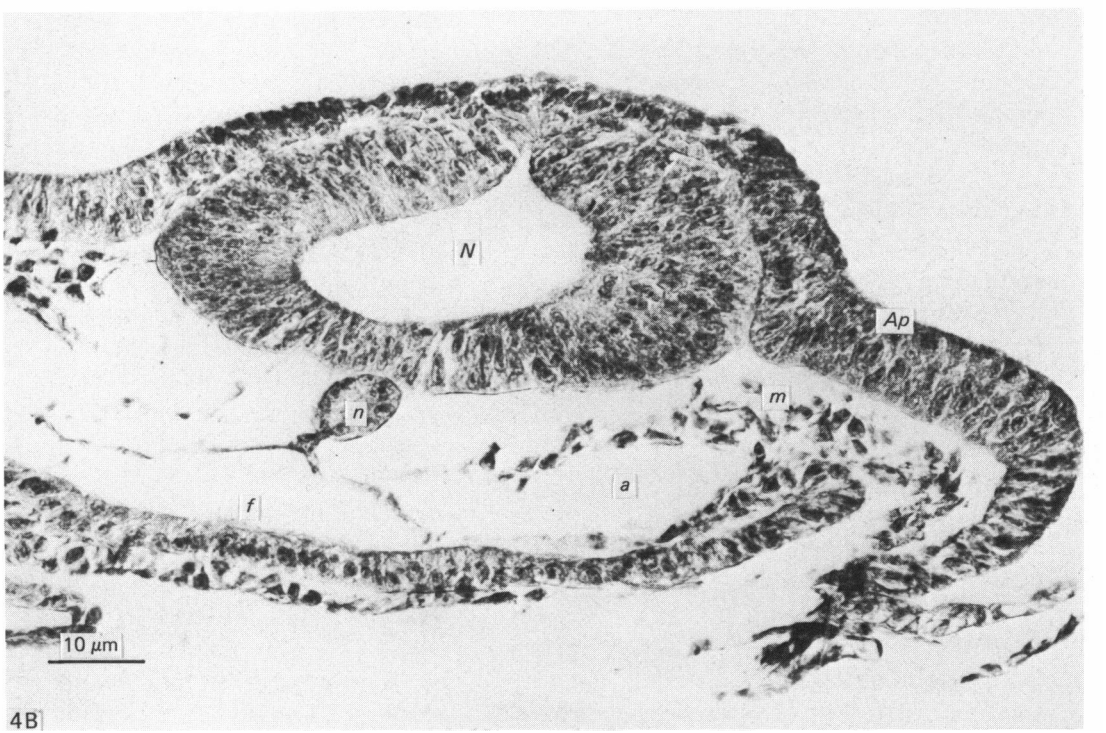


Fig. 4. Transverse sections at the auditory pit level in stage 12 embryos. (A) Control. (B) Exposed to pulse A, 13·9 μT. There is dorsoventral flattening of all organs and poorly developed vessels, foregut, and mesenchymal tissue. *N*, neural tube; *Ap*, auditory pit; *a*, aorta; *n*, notochord; *m*, mesenchyme; *f*, foregut. ×800.

(Table 4) were between 1·27 for the cephalic nervous system and 1·00 for somites. It was concluded that this intensity had no notable effect on embryonic development.

Exposure to 13·9 μT

In this group there were 32 exposed and 32 control eggs. As shown in Table 3, there was an increase in the number of abnormal embryos in the exposed group (53·1 %) as compared to controls (34·3 %), but the ratio, 1·54, ($0·25 > P > 0·1$) was not significant.

In exposed eggs, the frequency of non-developed embryos (15·6 %) was five times higher than in controls (3·1 %). Furthermore, the developmental stage reached by normal embryos was slightly delayed in the exposed sample (stage 10.5) when compared with controls (mean stage 11.5), corresponding to a difference of seven to eight hours. Heart and vessel abnormalities were significantly more frequent in exposed than in control embryos, with a ratio of 1·88 ($0·10 > P > 0·05$).

Microscopic examination of the 17 exposed embryos with heart and vessel abnormalities revealed that, in 14 cases, the heart was absent and vascularisation had not developed. Of these 14 cases, seven had not yet reached stage 8 (i.e. the stage of normal initiation of heart and vascular system organogenesis). In the other seven cases, however, embryos had reached beyond stage 8 and showed undeveloped heart and extra-embryonic vascularisation. The intra-embryonic vessels had poorly organised walls. One example is shown in Figure 4 where a transverse section at the auditory pit level in a stage 12 normal embryo (Fig. 4A) may be compared with a similar section of an exposed abnormal embryo of the same stage (Fig. 4B). These sections reveal a lack of endothelial organisation in the dorsal aortae and an underdeveloped foregut.

In another three embryos, the heart was displaced and abnormally twisted, with large blood islands but no vessels. In all these abnormal embryos, transverse sections showed a dorsoventral flattening of the organs (Fig. 4B) as previously observed in embryos exposed to an electromagnetic field of 1·0 μT intensity.

Exposure to 104 μT

The highest electromagnetic field intensity used (104 μT) was tested on 59 embryos and compared with 53 controls. The frequency of abnormal specimens was similar in the exposed (28·8 %) and control (24·5 %) groups (Table 3). Analysis of abnormalities in the different developing systems (Table 4) revealed no significant disturbances, contrasting with the effects of intensities seven times (13·9 μT) and one hundred times (1·0 μT) weaker.

Effects of pulse shape changes

All the experiments described above were performed with pulse shape A (Fig. 1), and in order to evaluate possible effects related to the use of different pulse shapes, three other types of pulse were tested.

Pulse B

This pulse was tested with a field intensity of 0·4 μT in 24 embryos and results were compared with 23 controls, as indicated in Tables 6 and 7. A strong teratogenic effect was demonstrated: while 34·7 % of controls were abnormal, 83·3 % of the exposed group presented abnormalities, giving a significant ratio of 2·40 ($0·005 > P$).

Table 7. *Abnormalities in systems of embryos of Table* 6

Intensity	0·4 μT		1·0 μT		1·0 μT	
	Exposed (N = 24)	Controls (N = 23)	Exposed (N = 30)	Controls (N = 29)	Exposed (N = 24)	Controls (N = 12)
Pulse shape	B		C		D	
CNS						
Abnormals	16	8	10	5	17	2
%	66·6	34·7	33·3	17·2	70·8	16·6
Ratio	1·91		1·93		4·26	
	(0·05 > P > 0·025)		(0·50 > P > 0·25)		(0·01 > P > 0·005)	
TNS						
Abnormals	16	8	8	3	17	2
%	66·6	34·7	26·6	10·3	70·8	16·6
Ratio	1·91		2·58		4·26	
	(0·05 > P > 0·025)		(0·25 > P > 0·10)		(0·01 > P > 0·005)	
Heart						
Abnormals	15	8	6	3	15	2
%	62·5	34·7	20·0	10·3	62·5	16·6
Ratio	1·80		1·94		3·76	
	(0·25 > P > 0·10)		($P \simeq$ 0·50)		($P \simeq$ 0·025)	
Vessels						
Abnormals	16	7	5	2	15	2
%	66·6	30·4	16·6	6·8	62·5	16·6
Ratio	2·19		2·44		3·76	
	(0·05 > P > 0·025)		(0·50 > P > 0·25)		($P \simeq$ 0·025)	
Somites						
Abnormals	17	7	9	3	15	2
%	70·8	30·4	30·0	10·3	62·5	16·6
Ratio	2·32		2·91		3·76	
	(0·01 > P > 0·05		(0·25 > P > 0·10)		($P \simeq$ 0·025)	

CNS, cephalic nervous system; TNS, truncal nervous system; N, number of embryos.

The frequency of non-developed embryos was twice as great in the exposed (16·6 %) as in the control (8·6 %) eggs.

Disturbances in the five systems were twice as numerous in the exposed as in the control embryos. Asymmetry was observed in eight of the 16 cases of cephalic nervous system abnormality in exposed embryos, while it was detected in only two of the eight abnormal controls. Other characteristic disturbances, seen in seven of 16 cases, were short truncal nervous systems with large open folds, grossly underdeveloped somites and an abnormal torsion of the embryo (Fig. 5A) which was not observed in either normal or abnormal controls.

Transverse sections of the embryos (Fig. 5B) showed extensive necrotic cells in the cephalic neural ectoderm and ectoderm, absence of basal membrane around the tissues, and diminution of acid glycosaminoglycans in extracellular regions. The truncal neural ectoderm was not organised and somites appeared as small groups of cells.

Exposure to this type of electromagnetic field also changed the timing of embryonic development. While normal controls reached a mean stage of 10.4 and abnormal controls reached 7.9, the exposed embryos advanced further, normal embryos reaching stage 11.7 and abnormals 8.8. This advance represented a difference of 8–9 hours in the case of the normal embryos and indicated the complexity of electromagnetic field effects.

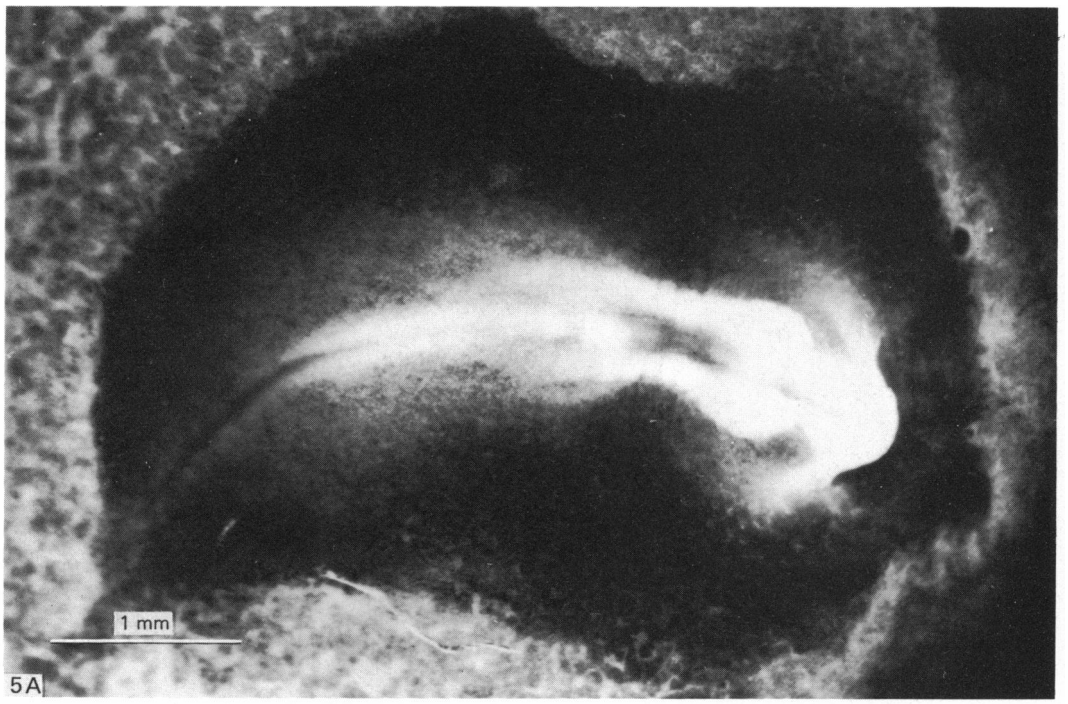
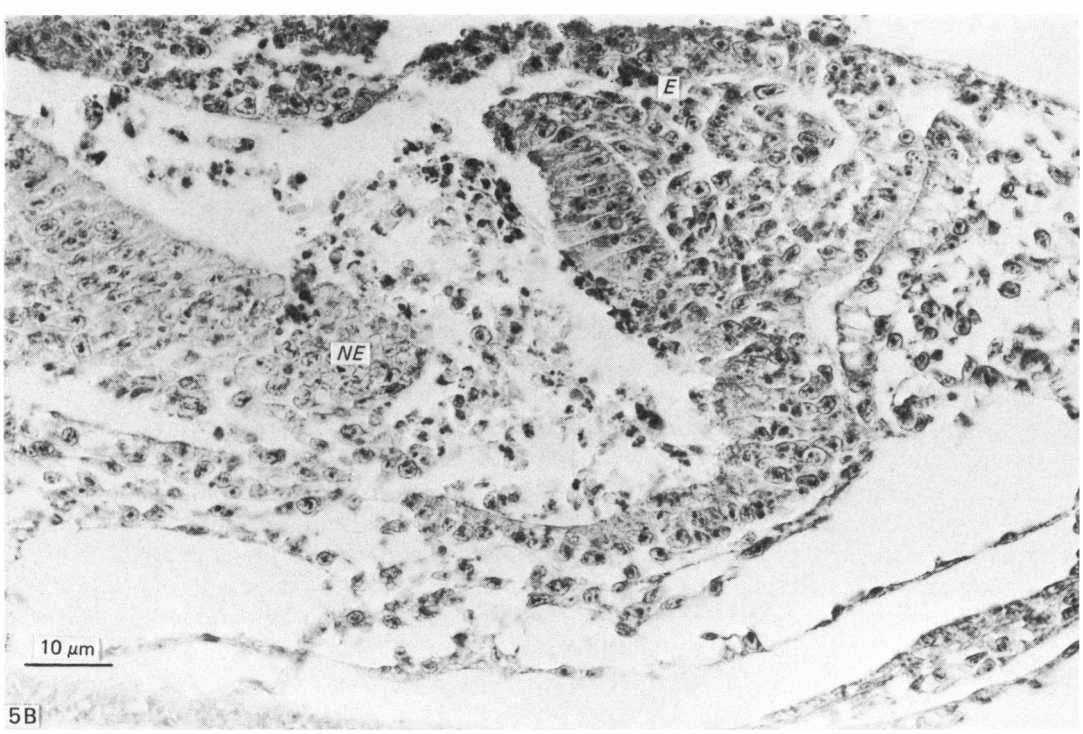


Fig. 5. Embryo exposed to pulse B, 0·4 μT. (A) Gross morphology showing abnormal torsion of the neural tube with large open folds. × 26. (B) Transverse section through the cephalic region showing extensive zones of necrotic cells in cephalic neural ectoderm (*NE*) and ectoderm (*E*). × 800.



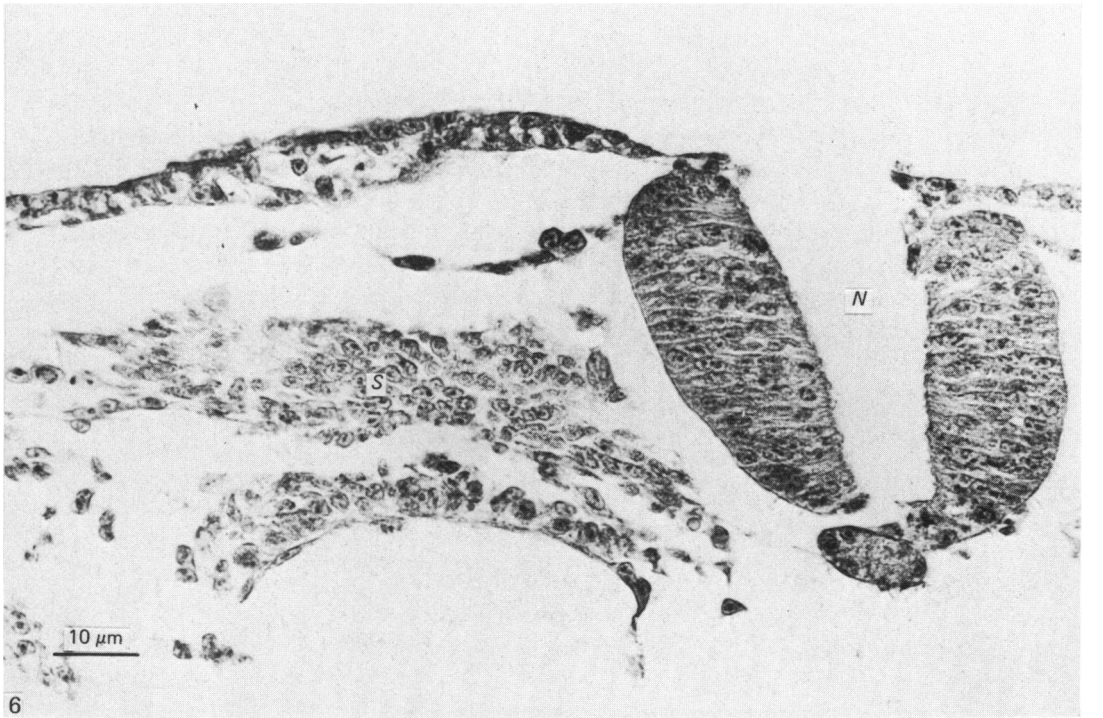


Fig. 6. Embryo exposed to pulse C, 1·0 μT. Transverse section through the fourth somite pair, showing open neural folds (*N*), poorly organised somites (*S*), and a general loss of cohesion. ×800.

Pulse C

A total of 30 embryos was exposed to pulse C with 1·0 μT and compared with 29 controls. Results, summarised in Table 6, indicated no significant increase in the frequency of abnormal embryos in the exposed group, with a ratio of 1·51 ($0{\cdot}50 > P > 0{\cdot}25$). The mean stage of development of normal embryos was similar in exposed and in controls (12.3 and 12.5), and the mean stage of abnormals was the same (11.7) in both groups.

Abnormalities were different in exposed and control embryos. Controls were affected in only one or two developing systems, while exposed embryos had abnormalities in three or four systems. Thus, abnormalities in all five systems (Table 7) were twice as frequent in exposed as in control embryos, with a ratio of 1·93 for cephalic nervous system and 2·91 for the somites. Although these results did not reach statistical significance, there were specific disturbances in the exposed abnormal embryos: in the 10 cases of malformation of cephalic nervous system, cephalic torsion and flexion were absent although these embryos reached a mean stage of 11.7. In the eight cases of a malformed truncal nervous system, six showed open folds and abnormal torsion. This effect was similar to that observed in the embryos exposed to pulse B with an electromagnetic field of 0·4 μT (Fig. 5A).

Transverse sections of the exposed abnormal embryos confirmed these morphological data, showing poorly organised somites. A loose cohesion of the organs and an increased tissue fragility were also noted (Fig. 6).

Pulse D

A total of 24 embryos was exposed to pulse D with a field intensity of 1·0 μT and compared with 12 controls. Results (Tables 6, 7) demonstrated the most powerful teratogenic effects of the experiments, as shown by increased numbers of abnormal embryos, with a ratio of 4·26 in cephalic and truncal nervous systems, and 3·76 in heart, vessels, and somites. In the exposed group, abnormal embryos were very small, and the development of the normal embryos was delayed, reaching a mean stage of 10.7 instead of 11.9 in controls. This corresponded to a delay of about 10 hours in development, indicating that electromagnetic fields disturbed the development of all exposed embryos.

Histological studies confirmed the importance of alterations in the exposed embryos. They presented a disorganised appearance, with abnormal and poorly developed cephalic nervous systems and truncal nervous systems which remained open, poorly formed hearts, altered somites, and a lack of vessel development (Fig. 7). Acid glycosaminoglycans in basal membranes and extracellular matrix were drastically reduced.

Electromagnetic field effects compared to pulse shapes

A summary of the results of the embryonic effects of electromagnetic fields with intensities of 0·4 and 1·0 μT, using pulses A, B and D, is shown in Figure 8. This illustrates the powerful effects of a field intensity of 1·0 μT using pulse D on the development of the five systems, which was far greater than the effects of the same intensity with pulse A. Figure 8 also indicates the trend of 'beneficial' effects when an intensity of 0·4 μT was used with pulse A, contrasting with its teratogenic influence when pulse B was employed.

DISCUSSION

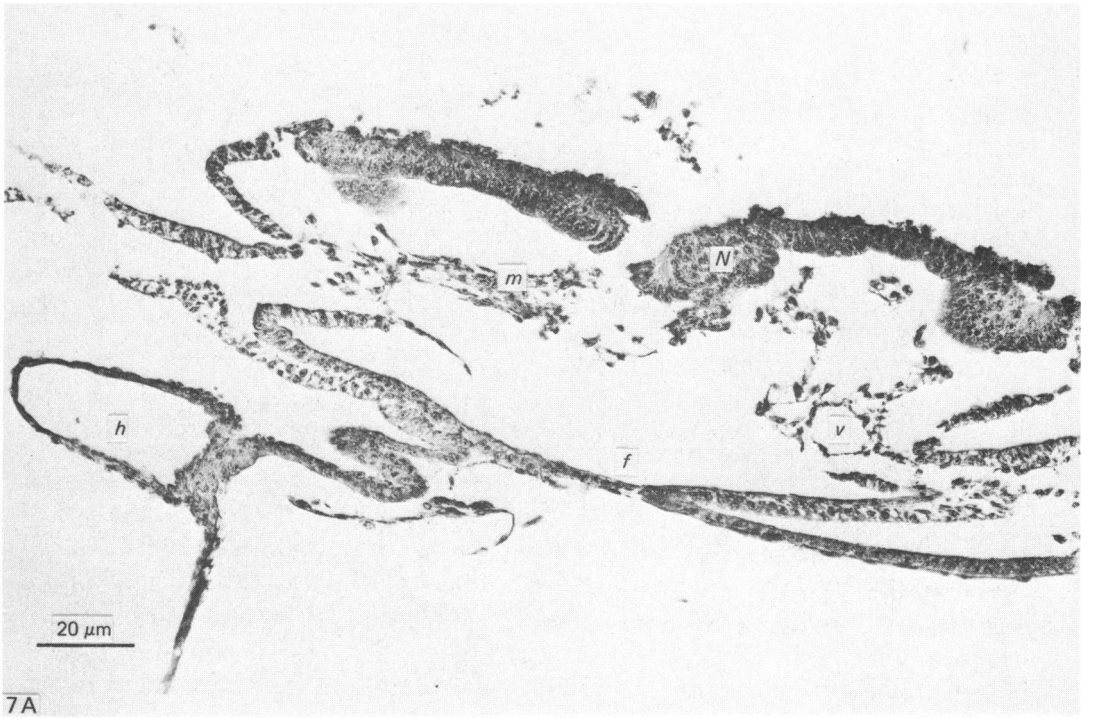
Effects of electromagnetic fields with pulse A characteristics

Teratogenic disturbances are observed with field intensities of 1·0 and 13·9 μT and not at the lower or higher intensities used, ruling out the possible influence of a rise in internal temperature on the embryonic tissues and confirming the existence of 'windows' in the teratogenic effects of electromagnetic fields (Adey, 1981; Delgado *et al.* 1982). Direct measurement of temperature during pulsating field exposure has been performed in another experimental series, confirming the absence of thermal changes.

Intensities of 1·0 and 13·9 μT have only slight, specific effects on development. Exposure to 1·0 μT disturbs the organogenesis of the truncal nervous system, and the developmental stage in abnormal exposed embryos (stage 12) is more advanced than in abnormal controls (stage 10.5). This advance corresponds to about 12 of the total 48 hours of incubation and, among other factors, may be related to an increase of mitotic activity. The frequency of abnormally thin and short neural tubes actually decreases in exposed embryos and very dense truncal folds are detected.

In recent experiments (M. A. Jimenez, in preparation), it has been observed that, after a brief exposure to electromagnetic fields at stages 7, 8 or 9, there is accelerated development accompanied by an increased mitotic index of the neuro-epithelium.

Goodman, Bassett & Henderson (1982) have demonstrated that 15 minutes' exposure of *Sciara coprofila* polytene chromosomes to pulsing fields can provoke a



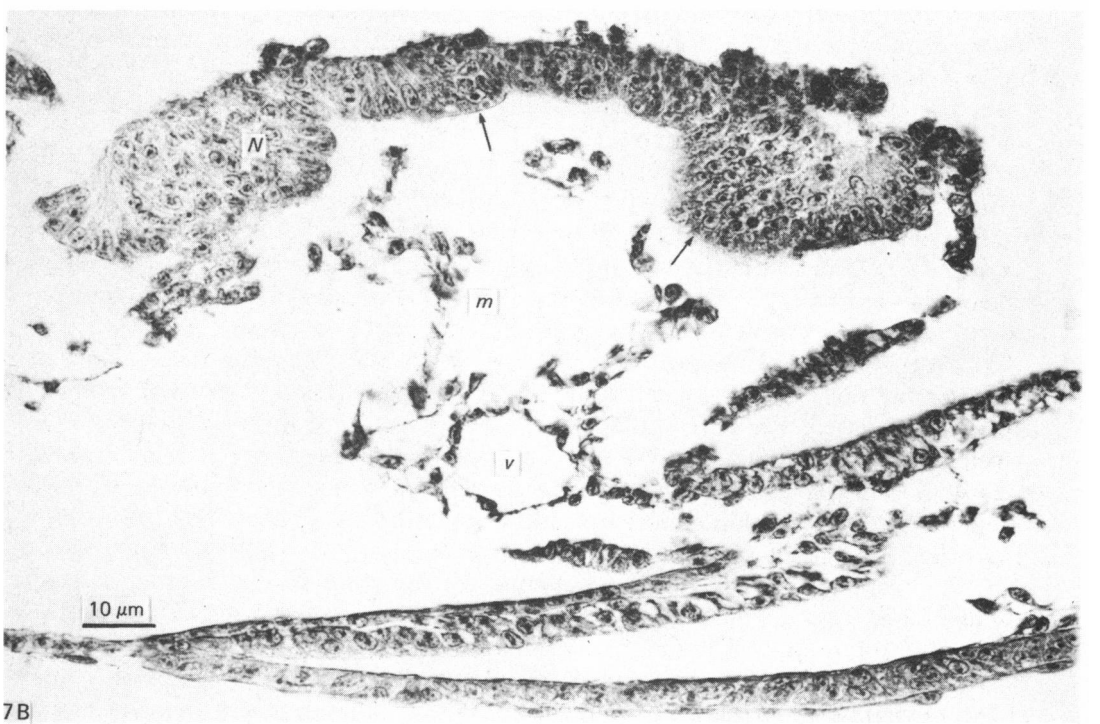


Fig. 7. Embryo exposed to pulse D, 1·0 μT. (A) Transverse section at the level of the heart showing underdeveloped and abnormal nervous system (*N*), foregut (*f*), heart (*h*), and vessels (*v*). ×400. (*B*) The notochord is absent, the basal membrane of the neural ectoderm (arrows) is discontinuous, and the somitic mesenchyme (*m*) is greatly reduced. ×800.

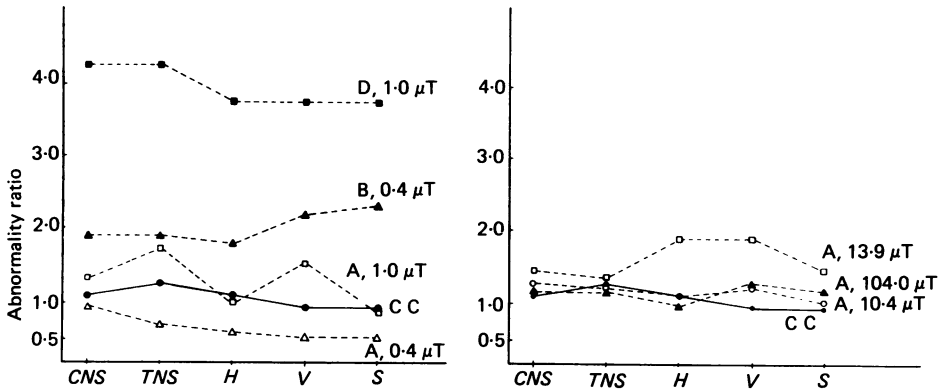


Fig. 8. Abnormality ratio in the cephalic nervous system (*CNS*), truncal nervous system (*TNS*), heart (*H*), vessels (*V*) and somites (*S*) in exposed embryos compared with their controls. The ratios were those indicated in Tables 2, 4 and 7. As shown, pulse D, 1·0 μT, caused the most teratogenic effects while pulse A, 0·4 μT, had a 'beneficial' trend on organic development. CC, Controls inside coils. A, B and D refer to pulse shapes.

fourfold increase in total RNA synthesis. A longer exposure reduces this effect, and RNA transcription diminishes to control levels or below. In the present experiments, exposure of chick embryos for two days to an electromagnetic field of 1·0 μT with pulse A could increase mitotic and metabolic activities during what may be a specific sensitive phase of development. In histological sections of the abnormal embryos, truncal neural tubes appear thicker and less organised than in controls at the same developmental stage (Fig. 3A, B). In these embryos, there is also a drastic reduction of alcian blue-stained components in the basal membrane of the truncal neuroepithelium and in the perinotochordal sheath.

It is known that acid glycosaminoglycans, which are components of tissue basal membranes and extracellular matrix (Kosher & Searls, 1973; Thesleff, 1978; Hay, 1978; Belsky, Vasan & Lash, 1980; Thesleff & Hurmerinta, 1981) play an important role in the regulation of cellular proliferation (Ohnishi, Ohshima & Ohtsuka, 1975; Roblin, Albert, Gelb & Black, 1975; Glimelius & Pintar, 1981), and also in organogenesis. Therefore the two types of histological alteration observed in neural tubes of the exposed embryos may be related to local changes in glycosaminoglycan synthesis, secretion or structure (Takaya, 1977).

Normal embryos of the exposed group reach the same stage of development as controls and no field effect is detected in the histological preparations. Thus it cannot be determined whether only a fraction of the exposed population is sensitive to the electromagnetic field or whether the apparently normal embryos might reveal alterations during their subsequent development. This question could be clarified by allowing the exposed embryos to develop until hatching and by performing physiological studies.

Organogenesis of the circulatory system is altered specifically at a field intensity of 13·9 μT without notable effects on the other systems. Transverse sections of malformed embryos show histological anomalies in the foregut which could be related to the specific abnormality of heart development (Manasek, 1976).

No relationship has yet been described between foregut tissue and vascular system organogenesis, but a common regulatory mechanism for development of the circulatory system (i.e. heart, intra-embryonic and extra-embryonic vessels and blood) may be altered by the application of an electromagnetic field. At least for vessel organo-

genesis, it may be proposed that the field acts upon membranes of the pre-endothelial cells, since the cell-to-cell contacts necessary for normal endothelialisation processes are altered in the abnormal embryos.

The normal surface coat of endothelial cells carries a net negative charge due to sialic acid. Bassett (1982) suggests that, in the bone 'gaps' of fracture non-union, the negative charge of fibrocartilage (which is rich in proteoglycans) prevents vascular invasion, so that electromagnetic fields could act by neutralising this electrical charge. In another series of experiments (M. A. Trillo, in preparation) stage 9 chick embryos have been treated *in vivo* with streptomyces hyaluronidase, which specifically hydrolyzes hyaluronic acid. After 10 hours, the embryos develop an enormous vascular system. Therefore, the disappearance of a major, negatively charged, component of the mesenchymal extracellular matrix may stimulate endothelial proliferation. In these experiments, however, the exposed embryos show normal glycosaminoglycan components, as demonstrated by a histological study performed with light microscopy, while abnormal cell-to-cell contacts are observed in the pre-endothelial cells. Therefore, rather than a direct action upon mesenchymal extracellular matrix, the electromagnetic field exposure could alter normal membrane processes of vascular wall differentiation, as in the Pilla (1974) model of living cell membranes.

A developmental delay in normal embryos and an increase in the frequency of non-developed embryos have also been noted in the group exposed to an electromagnetic field of 13·9 μT, indicating that all exposed embryos are affected. The biological response is either morphological, or produces a delay or arrest of the developmental programme.

Transverse sections of all abnormal embryos in groups exposed to field intensities of 1·0 and 13·9 μT (pulse A) show a common feature. A dorsoventral flattening is found, which is never observed in the normal embryos or in those exposed to other intensities, or in controls. While the explanation is not yet clear, the effect seems to be specific and typical.

Unlike fields of 13·9 μT, exposure to 10·4 μT has no effect on morphology or on the mean developmental stage attained by the exposed embryos. Chick embryo development appears to be very sensitive to slight variations in the intensity of the applied field.

Electromagnetic field effects with pulse forms B, C and D

Application of waveform B at an intensity of 0·4 μT has a significant teratogenic effect (Table 6). This intensity, which is 75 times less than the mean value of the Earth's static magnetic field, increases twofold the number of organic abnormalities as compared with controls, and accelerates embryological development. These results demonstrate the fine sensitivity of chick embryos to pulsating electromagnetic fields of extremely low frequency and intensity.

Comparison of field effects with pulses C and D at an intensity of 1·0 μT (Table 6) shows that the biological response is markedly diminished when high frequency noise is added to the magnetic pulse, indicating that embryological effects of pulsed electromagnetic fields are also related to the noise components of the waves. Exposure of eggs to pulse D at an intensity of 1·0 μT induces four times more abnormal embryos than in controls. This effect is similar to our previous findings with comparable electromagnetic field parameters (Delgado *et al.* 1982).

The following specific characteristics of abnormal embryos exposed to waveforms B, C and D may be emphasised. (1) Each abnormal embryo shows anomalies in

various systems, especially those exposed to a field of 0·4 μT, pulse B, and to 1·0 μT, pulse D (Table 7). (2) Cephalic flexion is absent in embryos which have developed beyond stage 11, while marked truncal torsions are present. (3) Truncal neural folds remain open. (4) Mesenchymal cell density decreases in the cephalic region and somites remain as small, poorly organised groups of cells. (5) Lack of cellular cohesion is observed in the different tissues. (6) Acid glycosaminoglycans are considerably reduced in basal membranes and in the extracellular matrix.

It is not yet known whether these effects result from metabolic changes occurring during gastrulation and/or organogenesis, or whether they are caused by direct action of the electromagnetic field on cell membranes and/or on glycosaminoglycan components.

Our results coincide partly with the teratogenic effects of electromagnetic fields on chick embryo development reported by Joshi *et al.* (1978) who describe embryonic abnormalities, including open neural tubes and diffuse somites, caused by exposure to a vertical and continuous electromagnetic field of 5000 Gauss. Similar malformations have been observed in the present study, but the intensity is 500,000 times lower. Hence pulsating fields seem to be far more effective than static fields. Findings presented in this study suggest that embryonic development can be more or less modified by varying the magnetic intensity or pulse shape.

SUMMARY

A total of 295 chick embryos was exposed during the first 48 hours of development to pulsed electromagnetic fields of 100 Hz and 0·4 to 104 microTeslas (μT), and findings were compared with those in 364 control embryos. General morphology was analysed and supplemented by light microscopy studies.

Exposure to electromagnetic fields with a pulse rise time of 100 μsec produced teratogenic changes when intensities of 1·0 and 13.9 μT were used but not with lower or higher intensities, demonstrating a 'window' effect and ruling out the possible influence of a rise in internal embryonic temperature. Exposure to an electromagnetic field of 1·0 μT specifically altered organogenesis of the truncal nervous system and drastically reduced the alcian blue-stained components, whereas with an intensity of 13·9 μT, there were abnormalities in the circulatory system and foregut, altering cell-to-cell contacts in the walls of developing vessels.

When embryos were exposed to intensities of 0·4 and 1·0 μT with 2·0 and 42 μsec pulse rise times, teratogenic effects were greater and alterations involved all developing systems. The most powerful effects were obtained with 1·0 μT and 42 μsec rise time.

The findings confirm the sensitivity of chick embryos to electromagnetic fields of extremely low frequency and intensity and indicate that pulse shape may be a decisive parameter determining strong, slight, or no modification of embryonic development. Mechanisms of action of electromagnetic fields are still unclear, but induced alterations in extracellular glycosaminoglycans could be a causal factor in the observed malformations.

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